neuronal migration, radial glial cells do not divide. After neuronal production and migration end, however, the radial glia enter a mitotic cycle, eventually differentiating into multipolar astrocytes. In lower vertebrates, radial glia has the capacity to form neurons but it is currently unclear whether radial glia or other types of glial precursors have the same capacity in mammals. Collectively, several studies suggest that may be only a small (and often reversible) transition between neuroepithelial stem cells and radial glia.

The use of cells for neural transplantation is well documented. Several studies have indicated that primary tissue from the developing ventral mesencephalon can give rise to dopaminergic neurons and supporting cells capable of survival, function, and therapeutic efficacy in Parkinson's patients. In addition, the transplantation of cultures containing neural precursor cells and stem cells can give rise all three major cell subtypes of the CNS, i.e. neurons, astrocytes, and oligodendrocytes. From these studies, there is a clear need in the art for cells capable of proliferating to make large numbers of cells as well as a capacity for neural differentiation in order to make the appropriate "adult" cells capable of integrating and restoring function to a diseased area in the CNS. Furthermore, over the past couple of decades, protein factors capable of protecting neural cells in the CNS from damage and capable of restoring function have bee discovered. From neuroprotection studies, it is evident that these protein factors may best work if delivered by gene manipulated cells placed in the area of disease. Thus, there is also a need in the art for transplantable neural cell lines capable of being gene modified in order to secrete protein factors locally. In addition, cell lines capable of making neurons and other neural lineages in a reproducible manner are useful screening targets to identify factors and drugs capable of influencing the CNS. Hence, there is a need in the art for neural cell lines for drug screening purposes. Last, with the human genome almost completely sequenced, there is a need for cells of neural lineages, which can be used to identify cDNA libraries to screen for gene function.

If glial precursor cells of the mammalian CNS could form neurons, astrocytes and perhaps other subtypes, a dividing pool of glial precursor cells could become a reliable source of large numbers of neural cells for the needs described above identifying several areas of industrial application. Preferably, cellular division in such glial precursor cells would be epigenetically

nigra. Cells are administered to the particular region using any method which maintains the integrity of surrounding areas of the brain, such as by injection cannula. Injection methods are exemplified by those used by Duncan *et al.*, 17 J. Neurocytology 351-361 (1988), and scaled up and modified for use in humans. Methods taught by Gage *et al., supra,* for the injection of cell suspensions such as fibroblasts into the CNS can also be used for injection of NS4 cells. Additional approaches and methods may be found in *Neural Grafting in the Mammalian CNS,* Björklund & Stenevi, eds. (1985).

NS4 cells administered to the particular neural region can form a neural graft, so that the cells form normal connections with neighboring neurons, maintaining contact with transplanted or existing glial cells, and providing a trophic influence for the neurons. Thus the transplanted NS4 cells re-establish the neuronal networks which have been damaged due to disease and aging.

Survival of the NS4 cell graft in the living host can be examined using various non-invasive scans such as computerized axial tomography (CAT scan or CT scan), nuclear magnetic resonance or magnetic resonance imaging (NMR or MRI), or positron emission tomography (PET) scans. Post-mortem examination of graft survival can be done by removing the neural tissue, and examining the affected region macroscopically and microscopically. Cells can be stained with any stains visible under light or electron microscopic conditions, more particularly with stains that are specific for neurons and glia. Particularly useful are monoclonal antibodies that identify neuronal cell surface markers such as the M6 antibody that identifies mouse neurons. Also useful are antibodies that identify neurotransmitters (such as GABA, TH, ChAT, and substance P) and to enzymes involved in the synthesis of neurotransmitters (such as GAD). Transplanted cells can also be identified by prior incorporation of tracer dyes such as rhodamine-labeled or fluorescein-labeled microspheres, fast blue, bisbenzamide, or retrovirally introduced histochemical markers such as the *lacZ* gene, which produces , α-galactosidase. Functional integration of the graft into the host's neural tissue can be assessed by examining the effectiveness of grafts on restoring various functions, including but not limited to tests for endocrine, motor, cognitive and sensory functions. Motor tests that can be used include those that measure rotational movement away from the degenerated side of the brain, and those that measure slowness of movement, balance, coordination, akinesia or lack of movement,

rigidity and tremors. Cognitive tests include various tests of ability to perform everyday tasks, as well as various memory tests, including maze performance.

The ability to expand NS4 cells *in vitro* for use in transplantation is also useful for *ex vivo* gene therapy. For instance, rat primary astroglial cells (Lundberg *et al.*, 139 Exp. Neurol. 39-53 (1996) or a human astroglial cell line (Tornatore *et al.*, 5 Cell Transplant 145-63 (1996)) have been transduced with the tyrosine hydroxylase gene and implanted in models of Parkinson's disease. More recently, astroglial cells for *ex vivo* gene therapy have also been derived from adult human cortex (Ridet *et al.*, 10 Hum. Gene Ther. 27 1-80 (1999)). Thus, NS4 cells provide an additional way to retrieve and expand astroglial cells for use as vehicles in *ex vivo* gene therapy trials.

Genetic Modification of NS4 Cells. Although the NS4 cells are non-transformed primary cells, they possess features of a continuous cell line. In the undifferentiated state, the NS4 cells continuously divide and are thus targets for genetic modification. In some embodiments, the genetically modified cells are induced to differentiate into neurons or glia by any of the methods described above.

The term "genetic modification" refers to the stable or transient alteration of the genotype of a NS4 cell by intentional introduction of exogenous DNA. DNA may be synthetic, or naturally derived, and may contain genes, portions of genes, or other useful DNA sequences. The term "genetic modification" as used herein is not meant to include naturally occurring alterations such as that which occurs through natural viral activity, natural genetic recombination, or the like.

Any useful genetic modification of the cells is within the scope of the present invention. For example, NS4 cells may be modified to produce or increase production of a biologically active substance such as a neurotransmitter or growth factor or the like. In one embodiment the the biologically active substance is a transcription factor such as a transcription factor that modulates genetic differentiation, *e.g.,* Nurr-1. In an alternative embodiment the biologically active substance is a non-mitogenic proliferation factor, *e.g.* v-myc, SV-40 large T or telomerase.

The genetic modification can be performed either by infection with viral vectors (retrovirus, modified herpes viral, herpes-viral, adenovirus, adeno-associated virus, and the like) or transfection using methods known in the art (lipofection, calcium phosphate transfection,

Tetanus toxin (available from Boerhinger Ingelheim) can be used to select out neurons. By varying the trophic factors added to the culture medium used during differentiation it is possible to intentionally alter the phenotype ratios. Such trophic factors include EGF, FGF, BDNF, CNTF, TGF, GDNF, and the like. For example, FGF increases the ratio of neurons, and CNTF increases the ratio of oligodendrocytes. Growing the cultures on beds of glial cells obtained from different CNS regions can also affect the course of differentiation.

The effects of the biological agents are identified based upon significant differences relative to control cultures with respect to criteria such as the ratios of expressed phenotypes (neurons, glial cells, or neurotransmitters or other markers), cell viability and alterations in gene expression. Physical characteristics of the cells can be analyzed by observing cell and neurite morphology and growth with microscopy. The induction of expression of new or increased levels of proteins such as enzymes, receptors and other cell surface molecules, or of neurotransmitters, amino acids, neuropeptides and biogenic amines can be analyzed with any technique known in the art which can identify the alteration of the level of such molecules. These techniques include immunohistochemistry using antibodies against such molecules, or biochemical analysis. Such biochemical analysis includes protein assays, enzymatic assays, receptor binding assays, enzyme-linked immunosorbant assays (ELISA), electrophoretic analysis, analysis with high performance liquid chromatography (HPLC), Western blots, and radioimmune assays (RIA). Nucleic acid analysis such as Northern blots and PCR can be used to examine the levels of mRNA coding for these molecules, or for enzymes which synthesize these molecules.

The factors involved in the proliferation of NS4 and the proliferation, differentiation and survival of NS4 cell progeny, and their responses to biological agents can be isolated by constructing cDNA libraries from NS4 cells or NS4 cell progeny at different stages of their development using techniques known in the art. The libraries from cells at one developmental stage are compared with those of cells at different stages of development to determine the sequence of gene expression during development and to reveal the effects of various biological agents or to reveal new biological agents that alter gene expression in CNS cells. When the libraries are prepared from dysfunctional tissue, genetic factors may be identified that play a role in the cause of dysfunction by comparing the libraries from the dysfunctional tissue with those

EXAMPLE 1

DISSOCIATION OF MURINE EMBRYONIC NEURAL TISSUE AND PROLIFERATION OF MURINE NS4 CELLS

Separate embryonic (E12-15) primary neural cultures were established by mechanical or enzymatic dissociation from the striatal anlage (lateral ganglionic eminence and medial ganglionic eminence) and grown in DMEM, 10% FCS, N2 supplement and EGF (20 ng/ml) for 4-20 passages. The cells grew adherently and phenotypes were analyzed using morphology and immunocytochemistry. For immunocytochemistry analysis, cells were fixed in 4% paraformaldehyde for 10 min. and exposed to primary and secondary antibodies according to well-established protocols. For neuronal differentiation, cells were switched to serum-free medium without EGF. After 1-7 days, cells were fixed and evaluated by morphology and immunocytochemistry. To evaluate the paternal origin of the differentiated progeny, cultures were established from embryonic transgenic mouse lateral ganglionic eminence and MGE expressing the receptor of the avian RCAS virus (called tv-a) under the control of the GFAP promoter. Thus, only cells that express GFAP can be infected by an RCAS-EGFP vector, which in turn marks the cells with green fluorescence. Therefore, any neuron that is derived from a cell that once expressed GFAP is rendered fluorescent.

After a couple of passages, the parental culture shows >95% GFAP and nestin immunoreactivity. In addition, the cultures express the radial glial marker RC2. After switching to serum-free medium and removing the EGF (differentiating condition), the cells change morphology and staining pattern to become as much as 36% neurons (as determined by morphology and ,beta-tubulin III). This finding could be reproduced with cells at least 15 passages old. The fact that the neurons had arisen from $GFAP^+$ parental cells was established beyond doubt by using the transgenic mouse cultures. Many of the beta-tubulin III immunoreactive cells also expressed EGFP indicating that they had been $GFAP^+$ at an earlier time point. Last, some cells retained their molecular identity and express transcription factors typical of differentiating neurons in the lateral ganglionic eminence (*e.g.* DLX and Meis2), showing that the cells are specified progenitors.

EXAMPLE 2

DISSOCIATION OF HUMAN EMBRYONIC NEURAL TISSUE AND PROLIFERATION OF HUMAN NS4 CELLS

Human first trimester CNS tissue was collected and the LGE and MGE were dissected out and mechanically dissociated and cultured in DMEM, N2 supplement, 10% FCS ("NS4 Complete Medium"), and EGF (20 ng/ml) or EGE and bFGF (20 ng/ml each). The tissues were incubated in 0.1% trypsin and 0.05% DNase in DMEM for 15-20 min at 37°C. Tissue was mechanically dissociated with a fire polished Pasteur pipette. Dissociated cells were plated at high density in tissue culture treated flasks without additional coating in "NS4 Complete Medium" and either EGF (20 ng/ml) or EGF and bFGF (20 ng/ml each). Cell cultures were housed in an incubator at 37°C, 100% humidity, 95% air/5% CO_2. When the cultures were confluent, they were passaged 1:3.

After several passages (>4), parental cells were investigated for their morphology and expression of GFAP, nestin, and beta-tubulin III. Similar to the mouse cultures (EXAMPLE 1), human cells were placed in serum-free medium without growth factors and the differentiation studied with morphology and immunocytochemistry. Adherent human cell cultures could be established in a similar manner to mouse cultures (EXAMPLE 1).

After several passages, a majority of cells showed GFAP and nestin inimunoreactivity and glial morphology. Both the EGF and EGF and bFGF stimulated cultures appeared similar in morphology and immunoreactive pattern. Upon switching to SFM and removal of growth factors, cells convened into a neuronal morphology in similar or possible even larger numbers than the mouse cultures and became immunoreactive to the neuronal marker, beta-tubulin III.

EXAMPLE 3

GLIAL METHODS

Lateral ganglionic eminence and medial ganglionic eminence sections were dissected from E13.5 or E15.5 embryos. The tissue pieces were incubated in 0.1% trypsin and 0.05% DNase in DMEM for 15-20 min at 37°C before mechanical dissociation and plating, at high density in tissue culture treated flasks without additional coating. Cells were expanded in DMEM F12 with N2 supplement (Gibco), glutamine (2 mM), antibiotics, 10% fetal calf serum (FCS), and EGF (20 ng/ml). When the cultures were confluent, they were passaged 1:3. Both neurons and glia were present in the initial cultures. However, by the 4th passage (P4), or after freezing and thawing, the cultures were devoid of cells possessing neuronal morphologies or expressing neuronal markers (*i.e.* beta-tubulin III). These cultures were highly enriched in cells expressing nestin as well as glial phenotypes (*i.e.* GFAP and RC2). In the case of LGE glial cultures, the cells were expanded extensively (passaged >25 times). These cultures expressed similar phenotypes to those passaged fewer times.

To generate neurones a medium-switch was performed on confluent cultures (3 days after plating and splitting) from the expansion medium to the same medium minus the serum and EGF. In some cases, a sequential switch was performed, where first serum was removed and then EGF a few days later. These cultures were kept in the serum-free medium (without EGF) for 4-8 days before fixation in 4% PFA and immunostaining for neuronal and glial markers (*e.g.* beta-tubulin III, GFAP, nestin and RC2).

EXAMPLE 4

LONGTERM EGF-STIMULATED CULTURES OF ATTACHED GFAP$^+$ CELL

In this EXAMPLE, cultures of dissociated cells prepared from lateral ganglionic eminence of the mouse embryonic day 15-17 (E15-17) forebrain were established in a medium including epidermal growth factor (EGF) and serum, to obtain propagating attached cultures with

a high content of astroglia-like cells. This EXAMPLE is to determine the long-term characteristics of cells cultured under these conditions. The cultures were passaged at confluency, and growth rate, morphology and phenotypic properties (*e.g.* GFAP immunoreactivity) were assessed after the subsequent passages. The cultured cells had the morphology of astroglial cells, with the vast majority of the cells immunoreactive for GFAP (around 90%), as well as for the intermediate filament marker nestin. The cells were also positive for the mouse-specific neural antibodies M2 and M6. The cells were negative when stained for the neuronal marker beta-tubulin III.

Dissociation. Lateral ganglionic eminence tissue was retrieved mainly from E15, but in a few cases also from E16-17, mouse embryos of timed pregnant mice. With the embryos immediately placed in a 1:1 mixture of Dulbecco's minimum essential medium (DMEM) and F12 (Gibco), the brains were removed, the cortex unfolded after a medial parasagittal cut and the underlying lateral ganglionic eminence dissected out bilaterally, using the method of Olsson *et al., 69* Neuroscience 1169-82 (1995). The tissue pieces, collected from one litter of embryos at a time, were then placed in a 0.1% trypsin (Worthington Biochemical Corporation)/0.05% DNase (Sigma) solution in DMEM/F12 and incubated for 20 min at 37^0C. Following rinses in DMEM/F12 with 0.05% DNase, the pieces were mechanically dissociated by repeated gentle trituration through the tips of two Eppendorf pipettes with decreasing diameters and centrifuged for 5 min at 600 rpm. The pellet was then resuspended and plated onto uncoated T75 flasks (Falcon), with a medium containing DMEM/F12 supplemented with 10% Fetal Bovine Serum (FBS; Sigma), EGF (20 ng/ml, human recombinant; R & D Systems), a defined hormone and salt mixture including 20 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 60 μM putresciene and 30 nM sodium selenite (all from Sigma Chemicals, St. Louis CO; *see,* Weiss *et al.*, 16 J. Neurosci. 7599-609 (1996)) and 1% AAS (antibiotic antimycotic solution; Sigma). The cultures were maintained at 37^0C with 95% air and 5% $C0_2$, with the medium changed every 2-3 days, and the cells passaged (or frozen down using DMSO and serum) at confluency.

In vitro cell cultures. The cultures were passaged at confluency, and growth rate, morphology and phenotypic properties (*e.g.* GFAP immunoreactivity) were assessed after the

set of cultures ("*In vitro* B"), the rate of division increased again after passage nine and thereafter stabilized at values equal to the initial culture period (FIG. 5).

Observations of in vitro cell cultures. From a few days after the dissection and initial plating, the cultures grew well, with smaller phase-bright cells and clusters of tightly aggregated cells, resembling so-called "neurospheres" (Reynolds & Weiss, 175 Dev. Biol. 1-13 (1996)), situated on top of dense islands of attached cells. Already after passage two, the cultures were more homogeneous, with small and tight islands of elongated attached cells, with short arm-like processes. Over the first few passages, the majority of the cells gradually became more flat and epitheloid, with a cubical or polygonal shape, and with fewer processes and more round and distinct nuclei, and thus resembling type I astroglial cells. During the slowly dividing passages (passages 5-8) the morphology of the cells changed, to a larger and more flat and round appearance, with long and thin processes and with cells less densely aggregated on the bottom of the flask. After passage 8 the cells reassumed the morphology of the early passages.

In control cultures from passage 11 without EGF, cells attached well after plating, but ceased to proliferate further, as followed over five weeks. The cell morphology also changed into a larger and more flat appearance, with long and thin processes extending from the cell body. Separate cultures were also prepared, without EGF already from the first plating and start of the cultures, and here no or very little growth was observed during the subsequent six weeks. These cells had a morphology resembling that of the passage 11 control cultures after EGF removal.

Immunoreactivity. GFAP-immunocytochemistry, performed after each of the first five passages and after passage 18, revealed that around 75% of the cells were $GFAP^+$ already after passage two, and with approximately 90% $GFAP^+$-immunoreactive cells after passage five, and also after passage 18 (FIG. 4). A similar proportions of the cells also expressed the intermediate filament nestin, both at the early and late passages. The mouse-specific neural markers M2 and M6 were also detected in the majority of the cells, overlapping with the GFAP and nestin immunoreactivities, but with a reduced expression of M2 at the later passages, and with M6 in general expressed at lower levels than M2. No or only occasionally beta-tubulin III^+ cells were detected at either passage five or 18. For immunocytochemistry, 100,000 cells were plated in uncoated 4-well plates (NUNC) after each passage, and after attachment fixed in 4%

paraformaldehyde (PFA). After rinses with potassium phosphate buffered saline (KPBS), the cultures were preincubated with 5% normal serum raised in the same species as the secondary antibody, in 0.02 M KPBS for 1 hr at room temperature (RT). Following incubation with primary antibodies (overnight at 4°C), the cultures were rinsed three times in 0.02 M KPBS (with 5% serum), and incubated with a biotinylated secondary antibody (2 hrs, 1(T), rinsed in KPBS and incubated with an avidin-biotin-peroxidase complex (Vectastain-Elite ABC Kit PK-6 100) using 3,3-diaminobenzidine as chromogen (25 mg/ml; Sigma).

Analysis of long-term attached cultures of GFAP$^+$ nestin$^+$ cells. We observed poor plating and growth in the control cultures initiated without EGF. We also observed a shift in growth rate during the extensive culture period, with about 25 passages over 7 months. The initially high rate of cell division gradually decreased over the first 8 passages, after which the cells increased their growth rate again, stabilizing at values equal to the first 4 passages, thus with features of a cell line (FIG. 5).

The early and late passage cells have many similarities in addition to the morphological and immunocytochemical characteristics presented here, such as immunoreactivity also for the radial glia marker RC-2 (FIG 3).

EXAMPLE 5

DIFFERENTIATION OF NS4 CELLS INTO NEURONS AND GLIA

To generate neurons, cells were proliferated as in EXAMPLES 1 or 2 on uncoated tissue culture plastic. Three days after plating, a medium switch was performed from the expansion medium to the same medium minus the serum and EGF. In some cases, a sequential switch was performed, where first serum was removed and then EGF a few days later. Cultures for indirect immunocytochemistry, were kept in the serum-free medium (without EGF) for 4-8 days before fixation in 4% paraformaldehyde in PBS. Following a 10 min fixation, coverslips were washed three times in PBS and immunostained for neuronal and glial markers (*e.g.* beta-tubulin III, GFAP, nestin, and RC2). Coverslips were incubated with primary antiserum in PBS/10% normal goat serum, 0.3% TRITON-X-100 for two hours at 37°C. Coverslips were washed 3x in PBS and

incubated with labeled secondary antibodies for 30 min at 37^{0}C. Coverslips were then washed 3x in PBS, rinsed with water and placed on glass slides. Between 17- 36% of the cells derived from cell cultures established from different dissections including human LGE, were neurons as determined by morphology and beta-tubulin III staining. To further examine the neuronal differentiation, ICC staining for GABA and calbindin were done indicating that most of the cells formed a GABA-ergic neuronal phenotype. Furthermore, the cells showed other evidence of retention of the striatal specification such as the expression of the marker DLX1 and MEIS2 but not PAX6 and NKX2.1, markers of cortical and MGE neurons respectively. The neuronal phenotype and function was further confirmed by electrophysiology which demonstrated electrochemical activity characteristic of neurons. During differentiation, many cells co-labeled with both beta-III-tubulin and GFAP. With further differentiation mature neuronal and astrocytic phenotypes and separate beta-II-tubulin and GFAP immunoreactivity were observed.

EXAMPLE 6

GENERATION OF NEURONS FROM MULTIPASSAGE GLIAL CULTURES AND CONFIRMATION OF GLIAL ORIGIN

Embryonic glial cells can be grown and expanded for several months in EGF and serum-containing medium, with a majority of the cells expressing high levels of GFAP and nestin, even after 25 passages. In this EXAMPLE, we used such glial cultures derived from the mouse telencephalon (E13.5 and E15.5); the LGE (lateral ganglionic eminence) and the MGE (medial ganglionic eminence). We show that after 4 to 25 passages, high numbers of neurons can be generated from these cells simply by removing serum and EGF from their culture medium.

The neurons we generate turn on, not only the neuron-specific marker beta-tubulin III, but also MEIS2 and DLX, transcription factors specific for the regions where the glia were dissected from.

To determine whether the neurons actually derive from glial cells we used cell cultures from the GFAP-tva mouse. These mice express the receptor for the RCAS-virus, tva, under the GFAP promoter (*see,* EXAMPLE 1). Thus, only GFAP expressing cells can be infected. After

clearly GFAP$^+$. Using a similar double labeling protocol, only occasional single mouse satellite-stained cells (0.5%) were also immunopositive for the neuronal antigen NeuN, either in the striatum or in the globus pallidus.

Discussion. Immunohistochemistry for M2 showed that the implanted cells developed an astroglia type I-like morphology, with a distribution overlapping that obtained by mouse-satellite DNA *in situ* hybridization (Brüstle *et al.*, 15 Neuron 1275-85 (1995)). Astroglia-like M2$^+$ cells were also positive for M6. No axonal projections were found to emanate from the implanted cells, and only occasional mouse-satellite DNA$^+$ cells could be labeled also with the neuronal marker NeuN. Thus, the implanted cells can survive and integrate well, and acquire an astroglial phenotype after implantation, both when grafted into the neonates and into the adult (lesioned and intact) recipient brains.

These findings from the grafts placed into the neonates and from the adult recipients are in agreement with previous work with grafts of primary ganglionic eminence tissue implanted into rat hosts of different developmental stages (Olsson *et al.*, 79 Neuroscience 57-78 (1997)).

The astroglial nature of the implanted cells was further evidenced by the finding that around 75% of the implanted cells (mouse-satellite DNA$^+$) were GFAP$^+$ in the host globus pallidus. In the striatum, the number of double labeled cells was lower, but also the overall GFAP-staining of the host brain was lower in that region. Interestingly, the pattern of GFAP-immunoreactivity in the grafted cells was thus regionally similar to that of the surrounding host brain.

The migration of astroglial cells from primary tissue-grafts is region specific, and thus dependent on from where the tissue is dissected (Gates *et al*, 84 Neuroscience 10 13-23 (1998)). Questions regarding the regional specificity of astroglial cells, could also be further addressed by growing relatively pure populations of astroglial cells from different CNS regions using the present or a similar culture protocol.

Importantly, no tumors were formed when implanting late passage cells, even though the cells showed a high growth rate *in vitro*. Although the late passage cells seemed to survive less well than the early passage ones, also the late passage cells had a clear astroglial morphology and showed similar migration patterns when grafted into the neonatal recipients.